OK TO ENTER: /S.H./

Docket No.: 60384(71699)
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Olga N. Kovbasnjuk et al.	
Application No.: 10/539,212	Confirmation No.: 2349
Filed: June 17, 2005	Art Unit: 1643
For: TREATMENT OF METASTATIC COLON CANCER WITH B-SUBUNIT OF SHIGA TOXIN	Examiner: S. J. Huff

AMENDMENT

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Final Office Action dated April 9, 2010, please amend the above-identified U.S. patent application as follows:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 4 of this paper.

AMENDMENTS TO THE CLAIMS

1. (Previously Presented) A method of reducing, or inhibiting invasiveness and metastasis of tumor cells in a subject, wherein the tumor cells produce Gb_3, comprising administering to the subject a therapeutically effective amount of a Stx1B-subunit of Shiga toxin.

2. (Original) The method of claim 1, wherein the tumor cells are colon tumor cells.

3. (Previously Presented) The method of claim 1, wherein the tumor cells are from a tissue selected from the group consisting of: colon, lung, brain, skin, ovary, pancreas, liver, stomach, bladder, bone, testicle, uterus, adipose tissue, throat, kidney, tongue, pituitary gland, thyroid, lymphoid tissue, eye, and cervix.

4. (Cancelled)

5. (Cancelled)

6. (Previously Presented) The method of claim 1, wherein the therapeutically effective amount of the Stx1B-subunit of Shiga toxin is administered prior to the onset of metastasis by the tumor cells.

7. (Previously Presented) The method of claim 1, wherein the therapeutically effective amount of the B-subunit of Shiga toxin is administered subsequent to the onset of metastasis by the tumor cells.

8. (Previously Presented) The method of claim 1, further comprising administering to the subject a therapeutically effective amount of radiation.

9. (Previously Presented) The method of claim 1, further comprising administering to the subject a therapeutically effective amount of at least one chemotherapeutic agent.

10. (Cancelled)

11. (Previously Presented) The method of claim 1, wherein the subject is a human.

12. (Previously Presented) The method of claim 1, wherein the Stx1B-subunit of Shiga toxin is conjugated to a therapeutic moiety.

13. - 17. (Cancelled)

18. (Currently Amended) A method of reducing, or inhibiting invasiveness and metastasis of colon tumor cells in a subject, wherein the tumor cells produce Gb_3, comprising administering to the subject a therapeutically effective amount of a Stx1B~~B~~-subunit of Shiga toxin.

REMARKS

Claims 1 – 3, 6 – 9, 11, 12 and 18 are pending in the application. Claims 5, 6, 10 and 13-17 have been previously cancelled. Claim 18 has been amended. No new claims have been added. No new matter has been added.

Any cancellation of the claims should in no way be construed as acquiescence to any of the Examiner's rejections and was done solely to expedite the prosecution of the application. Applicant reserves the right to pursue the claims as originally filed in this or a separate application(s).

Claim Rejections Withdrawn

The Examiner has withdrawn the rejection to claims 1 – 3, 6 – 9, 11 – 12 and 18 under 35 USC §112, second paragraph.

Claim Rejections 35 USC §103(a)

Claims 1 – 3, 6 – 9, 11 – 12 and 18 have been rejected under 35 USC §103(a)as being unpatentable over the combination of Marcato et al. (Infection and Immunity vol. 70 p.1279 (2002) in view of LaCasse et al.(Blood vol. 88 p.1561 (1995)) and Strockbine et al. (J Bacteriology vol. 170 p.1116), Accession Number 2002:397002, Green (US 2002/0081307) and Applicant's admission on page 6, lines 1 – 2 of the specification. (Office Action, p.3). Applicants respectfully disagree.

The present claims recite a method of reducing, or inhibiting invasiveness and metastasis of tumor cells in a subject, wherein the tumor cells produce Gb3, comprising administering to the subject a therapeutically effective amount of a Stx1B subunit of Shiga toxin.

As discussed in the previous response, Applicants have **particularly identified Stx1B for use in the methods as claimed**. Applicants teach that there are a number

of Shiga toxin variants and subunits, for example at page 6, beginning at line 30 of the present disclosure:

> The sequences of numerous Shiga toxin variants and subunits are known in the art. For example, the Shiga toxin 1 B-subunit from the E. coli O157:H7 strain is set forth in GenBank Accession Nos. 32400300 and 32400303, the Shiga toxin 2 B-subunit from the E. coli O157:H7 strain is set forth in GenBank Accession No. 13359150, the Shiga toxin 1 A-subunit is set from the E. coli O157:H7 strain is set forth in GenBank Accession Nos. 32400299 and 32400302, and the Shiga toxin 2 A-subunit from the E. coli O157:H7 strain is set forth in GenBank Accession No.15718405.

Among all of these variants and subunits, **Applicants have particularly identified Stx1B for use in the methods as claimed.**

Further, Applicants point out that in the present work, recombinant Stx1B has been used to demonstrate the effects of reducing, or inhibiting invasiveness and metastasis of tumor cells, as claimed. In Applicant's work, recombinant B subunit of Shiga toxin (Stx1B) was obtained from the GRASP Center, New England Medical Center (see, e.g. p. 36, line 4-5). Applicants show that the recombinant B-subunit alone causes apoptosis in human colon cancer cells (see, e.g. Example 7 at p. 43). In Example 8, on pages 43 - 44, Applicants show that Stx1B selectively causes apoptotic death in cells expressing Gb3. It is well known to one of skill in the art that the Stx holotoxin has very different effects from the individual subunits. It is well known to one of skill in the art that the holotoxin contains both the enzymatic Stx A and five Stx B subunits, and that the A subunit is well-known to be toxic.

None of the references cited by the Examiner, taken alone or in combination, teaches or suggests the present invention as claimed.

The test of obviousness requires that one compare the claimed "subject matter as a whole" with the prior art "to which said subject matter pertains" 35 U.S.C. § 103(a). To establish a prima facie case of obviousness, three criteria must be met. First, a suggestion or motivation to modify the reference or combine reference teachings must be present in the references or in the general knowledge present in the art. Second, there must be a reasonable expectation of success. Finally, the prior art reference must

teach or suggest all the claim limitations. M.P.E.P. 2143. The burden is on the Examiner to show that the references expressly or impliedly suggest all of the claim limitations. M.P.E.P. 2142. "There are three possible sources for a motivation to combine references: the nature of the problem to be solved, the teachings of the prior art, and the knowledge of persons skilled in the art." In re Rouffet, 149 F.3d 1350, 1357. In the absence of some teaching or suggestion to combine, no prima facie case of obviousness can be established, and the rejection is improper and must be withdrawn. In re Fine, 837 F.2d 1071, 1074.

In the present case, the references cited by the Examiner fail to provide the requisite motivation to combine, fail to provide a reasonable expectation of success, and fail to teach or suggest all of the claim limitations. Each of the references cited by the Examiner in support of the obviousness rejection is considered below.

The Marcato et al. reference is directed to the use of the cloned shiga toxin B (Stx2 B) subunit to induce apoptosis in Burkitt Lymphoma B-cells. Applicants attach a Retraction of this work by Mercato (Mercato et al., Infection and Immunity, Aug. 2003, p.4828; provided herein), where Mercato et al. indicate that

> we discovered that additional preparations of cloned Shiga toxin 2 (Stx2) b subunit lacked apoptogenic activity in Ramos Burkitt's lymphoma B cells…We discovered that the Stx B subunit preparations used in our study contained previously undetected Stx2 holotoxin. **Since this contaminating Stx2 holotoxin was likely responsible for the apoptogenic activity we attributed to the Stx2 B subunit** in this article, we retract the conclusion that the Stx2 B subunit, absent any subunit activity, initiated apoptosis in Ramos cells. **This new finding does not alter our conclusions related to the lack of apoptogenic activity in the Stx1 B subunit preparation**…

As indicated above by Mercato et al., the Examiner's argument on p. 6 of the Office action, that "Applicant's misplaced assertion that apoptosis was not observed in the A subunit free preparations of the Stx1 B pentamer" is incorrect. **The Stx 1B**

subunit does not show apoptotic activity. As described by Mercato et al., above, there is clearly a difference in activities between the holotoxin and recombinant Stx subunits. As discussed, the holotoxin contains both the enzymatic Stx A and five Stx B subunits, and, as shown by Mercato et al. above, the A subunit is well-known to one of skill in the art to be toxic.

Accordingly, the teachings of Marcato would not lead one of skill in the art to choose StxB1 as an apoptosis inhibitor in the claimed methods.

Nowhere in the Marcato reference is there teaching or suggestion of a method of reducing, or inhibiting invasiveness and metastasis of tumor cells in a subject, wherein the tumor cells produce Gb3, comprising administering to the subject a therapeutically effective amount of a Stx1B subunit of Shiga toxin as claimed.

None of the LaCasse, Strockbine or Greene references cure the defects of the Marcato reference. None of the references, alone or in combination, teach or suggest a method of reducing, or inhibiting invasiveness and metastasis of tumor cells in a subject, wherein the tumor cells produce Gb3, comprising administering to the subject a therapeutically effective amount of a Stx1B -subunit of Shiga toxin.

As previously discussed, the LaCasse reference is directed to the use of shiga like toxin (SLT-1) in human bone marrow (BM) purging. LaCasse uses Shiga Like Toxin (SLT-1) which kills cells by inhibiting protein synthesis. (p.1561). The purpose of the study described by LaCasse "was to establish the potential of a natural toxin (SLT-1) in purging B-cell lymphomas from BM." (p.1563).

LaCasse does not teach or suggest a method of reducing or inhibiting invasiveness and metastasis of tumor cells in a subject using Stx1B.

The Examiner argues that "LaCasse et al disclose treatment of human B cell lymphoma from bone marrow in mice using Shiga-like toxin 1 (and) also discloses that the toxin was administered after the cancer is present." (Office Action, p.4). The Examiner argues further that "(o)n page 6 of the specification, Applicant admits the toxins are known to bind to Gb3 expressing cells, therefore it is expected that the cells of the reference are Gb3 expressing cells." (Office Action, p.4).

Further, Applicants point out that LaCasse purifies the B-subunit of SLT-1 from an E.coli culture as described by Ramotar et al. (Biochem J. 1990. 272. 805-811; attached). It was well-known to one of skill in the art at the time of filing that *E.coli* was not a reliable system for producing the B-subunit. Referring to p. 1990 of the Ramotar reference, Ramotar teach that "(i)n five separate experiments, the first two polymixin B extracts yielded SLT-1B at quantities of 80+/-58 and 48+/-25 µg/ml of the original culture, respectively…(t)he mean total yield from polymixin B extracts and pellet wash was 160+/-79 µg/ml of culture." These are terrible yields. Moreover, this result demonstrates that 50% in total yield of Stx1 B was unknown/ uncharacterized substances, which can produce many unknown side effects in cancer models, including apoptosis. Further, Figure 2, lane D, shows that there was no detection of the purified B-subunit of Stx-1. Given these results, it is not clear what LaCasse et al. were actually using in their experiments. It was known in the art at the time of filing that *Vibrio cholera* was the organism of choice for producing shiga toxin. Applicants attach the Acheson reference (Infection and Immunity, Jan. 1996, p.355 – 357; provided herein), that teaches, using *V. cholera*, large amounts of recombinant SLT-1B subunit can be produced in vitro, for example, over 10 mg of SLT-1B have been purified per liter of culture. (p.356).

It would not have been obvious to one of ordinary skill in the art that StxB subunit can also be used to inhibit apoptosis in vivo, as argued by the Examiner on page 5 of the Office Action. Thus, the teachings of Marcato would not motivate one to use Stx1B in place of SLT-1, as taught by LaCasse. The teachings of the cited art, when combined, do not result in the claimed invention.

Accordingly, Applicants request that the rejection be withdrawn and the claims allowed.

CONCLUSION

In view of the above amendment, applicant believes the pending application is in condition for allowance.

Dated: September 9, 2010

Respectfully submitted,
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Protective Immunity to Shiga-Like Toxin I following Oral Immunization with Shiga-Like Toxin I B-Subunit-Producing *Vibrio cholerae* CVD 103-HgR

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This study addresses a mechanism for inducing systemic immunity to Shiga-like toxins by oral administration of a Shiga-like toxin I B-subunit-expressing *Vibrio cholerae* vaccine strain [CVD 103-HgR(pDA60)]. Two sets of three rabbits were given either CVD 103-HgR or CVD 103-HgR(pDA60) orally. All rabbits immunized with CVD 103-HgR(pDA60) developed neutralizing serum antibodies to Shiga-like toxin I. None of the controls developed such antibodies.

Shiga-like toxins (SLTs) (also known as verotoxins), which were named after the prototype toxin produced by *Shigella dysenteriae* type 1, are produced by *Escherichia coli* and are well-documented causes of morbidity throughout the world. SLT-producing bacteria are now a major cause of acute renal failure in children in the United States. These bacteria have been associated with bloody and nonbloody diarrhea and with systemic complications such as hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (8, 12, 16). Over 80 serotypes of SLT-producing *E. coli* have been identified in humans, and the production of SLTs is the one feature that they all share. Free SLTs are detectable in the stools of patients infected with SLT-producing *E. coli* (2, 13). These facts, combined with the proven in vitro cytotoxic effects of SLTs on intestinal epithelial and various endothelial cells, which are considered major targets of the toxins in vivo (11, 18, 21), suggest that SLTs are critical virulence factors in both gastrointestinal and systemic disease.

SLTs can be broadly divided into two groups: SLT-I, whose amino acid sequence differs by only three nucleotides from that of the Shiga toxin produced by *S. dysenteriae* type 1 strains, and SLT-II, which is about 60% homologous with SLT-I at the amino acid level (10). All SLTs are heterodimeric nonglycosylated proteins consisting of an enzymatic A subunit, which inactivates the 60S ribosomal subunit and inhibits protein synthesis, and a pentamer of binding B subunits (5, 9, 19, 22). We previously cloned and hyperexpressed the SLT-I B subunit in both *E. coli* and *Vibrio cholerae* (1), and expressed the SLT-II B subunit in *E. coli* (3).

Currently the only therapy available for patients infected with SLT-producing *E. coli* or suffering from the systemic effects of the toxins, such as hemolytic uremic syndrome, is supportive. While control measures, in relation to food preparation and cooking, are the most appropriate means of preventing SLT-induced disease, there is a need for alternative strategies such as the development of active therapy and vaccines which could prevent SLT-related hemolytic uremic syndrome. It is clear from animal studies that the presence of serum antibodies to the toxins offers protection against toxin-induced disease (7, 23). Purified SLT-I B subunit has been shown to induce a protective immune response when given parenterally to rabbits (17) and may be a suitable antigen for use as a human vaccine to prevent the local and systemic complications caused by SLT-producing *E. coli*.

In view of current trends towards enteral vaccine delivery and multicomponent vaccines, the objective of the present study was to use the *V. cholerae* vaccine strain (CVD 103-HgR) to present SLT-I B subunit to the gut mucosa by the oral route and then to determine whether an immune response to the foreign antigen was elicited. CVD 103-HgR is a recombinant derivative of *V. cholerae* O1 strain 569B in which 94% of *ctxA1* has been deleted and a 4.2-kb fragment encoding resistance to mercury has been inserted into the *hylA* locus (15). The SLT-I B-subunit-expressing plasmid (pDA60) was constructed by subcloning a portion of the *slt1* gene containing DNA encoding the entire SLT-I B-subunit gene and the carboxy-terminal 18 amino acids encoded by the A subunit gene into pKK223-3 (Pharmacia LKB Biotechnology, Piscataway, N.J.) under the control of a *tac* promoter. pDA60 was inserted into CVD 103-HgR by electroporation. Six male New Zealand White rabbits (approximately 7 weeks old) were anesthetized with xylazine and ketamine after a 12-h fasting period. The rabbits were then each given 50 mg of cimetidine intravenously. After 15 min, a stomach tube was inserted in each rabbit and 10 ml of a 10% sodium bicarbonate solution was administered, followed by the bacterial inoculum (10^{10} to 10^{11} organisms); 30 min later they were each given 1 ml of tincture of opium intraperitoneally. Three rabbits received the parental strain, CVD 103-HgR, and three were given CVD 103-HgR(pDA60). All rabbits were given a second oral inoculation of the same bacteria 20 days after the first inoculation. Blood was drawn from the rabbits prior to the experiment, 12 days after the first inoculation, and 14 days after the second inoculation. Approximately 5 weeks after the second inoculation, enterotoxicity responses to both cholera toxin and various doses of SLT-I were assessed in the six rabbits, as previously described (14).

Sera were tested for the presence of immunoglobulin G antibodies to SLT-I B subunit by coating enzyme-linked immunosorbent assay (ELISA) plates (Nunc Maxisorp) with 5 μg of recombinant SLT-I B subunit per ml (1). Various dilutions of serum were added, bound antibodies were detected with a

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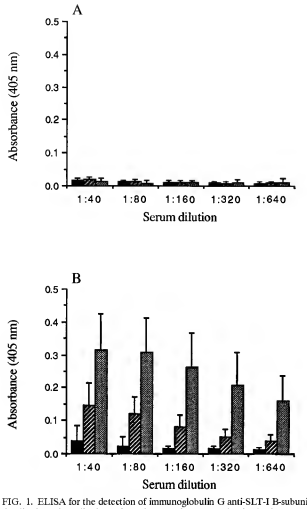

FIG. 1. ELISA for the detection of immunoglobulin G anti-SLT-I B-subunit antibodies in various dilutions of rabbit serum from control animals given CVD 103-HgR (A) and animals given CVD 103-HgR(pDA60) (B). Blood was tested prior to immunizations (solid bars), after the first immunization (hatched bars), and after the second immunization (cross-hatched bars). Data are means + standard errors of the means for three rabbits in each group.

goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate followed by Sigma 104 phosphatase substrate, and the results were expressed as A_{405}. Having already established that a concentration of 1 ng of SLT-I per ml killed approximately one-half of the HeLa cells in a well of a 96-well plate (data not shown), we determined the neutralizing capacity of the serum antibodies to SLT-I by incubating various concentrations of serum with SLT-I (1 ng/ml) for 1 h at room temperature before adding the toxin-serum mixture to HeLa cells grown in 96-well plates. Following overnight incubation at 37°C (5% CO_2), the plates were washed, the remaining attached cells were stained with crystal violet as described (6), and the results were expressed as percent HeLa cell survival compared with survival of non-toxin-treated control cells.

We have previously found that *V. cholerae* CVD 103-HgR(pDA60) produces large amounts of SLT-I B subunit in vitro, and we have been able to purify over 10 mg of SLT-I B subunit per liter of culture (4). The results from the present experiments demonstrate that there was little difference among preimmune sera from the animals and that none of the animals immunized with CVD 103-HgR developed an antibody response to SLT-I B subunit. In contrast, there was evidence of antibodies to SLT-I B subunit in the animals immunized with CVD 103-HgR(pDA60) after the first immunization. The level of antibody, as determined by the ELISA, rose further after the second immunization (Fig. 1). Similar results were observed in the neutralization experiments (Fig. 2). In contrast, there was a clear serum vibriocidal antibody response in all six rabbits after the second immunization. The titers in the three rabbits given CVD 103-HgR were 160, 320, and 20,480, and the titers in the three given CVD 103-HgR(pDA60) were 160, 320, and 640. None of the rabbits had antibodies to the B subunit of cholera toxin in the preimmune serum; however, five of the six rabbits, including all those given CVD 103-HgR(pDA60), developed a significant antibody response to the B subunit of cholera toxin after the second immunization. To maximize the data from each animal, we also determined the effects of immunization on toxin-induced intestinal fluid secretion (20). Ileal loops were constructed and inoculated with buffer as a negative control, with cholera toxin as a positive control, or with various doses of SLT-I. Of the six rabbits, two (both of

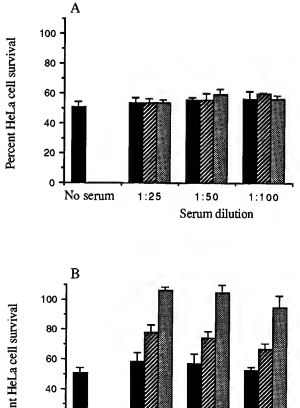

FIG. 2. Capacity of various dilutions of rabbit serum to neutralize the effects of SLT-I on HeLa cells. Results for control animals given CVD 103-HgR (A) and animals given CVD 103-HgR(pDA60) (B) are shown. Blood was tested prior to immunizations (solid bars), after the first immunization (hatched bars), and after the second immunization (cross-hatched bars). Data are means + standard errors of the means for the three rabbits in each group. HeLa cell survival is calculated as a percentage of the survival of cells grown in the absence of toxin (high survival values denote low levels of toxin).

which received CVD 103-HgR) died during the experiment. The four remaining animals [one given CVD 103-HgR and three given CVD 103-HgR(pDA60)] were sacrificed 18 h after inoculation. There was little difference in response to cholera toxin among the four animals (3.5, 2.8, 3.2, and 3.0 ml of fluid per cm of ileum in the rabbit given the parental strain and the three immunized with the SLT-I B-subunit-producing strain, respectively). The loops injected with 1 μg of SLT-I produced 1.4, 0.3, 0.0, and 0.6 ml of fluid per cm of ileum in the rabbit given the parental strain and the three immunized with the SLT-I B-subunit-producing strain, respectively. Administration of 100 ng of SLT-I resulted in 0.8, 0.2, 0.0, and 0.0 ml of fluid per cm of ileum in the rabbit given the parental strain and the three immunized with the SLT-I B-subunit-producing strain, respectively. While the number of animals used in the study is too small for one to draw any definitive conclusions, the loop experiments suggest that the three animals given CVD 103-HgR(pDA60) were somewhat protected from the enterotoxic effects of SLT-I compared with the single control. The fact that all three animals with antibodies to SLT-I B subunit survived the SLT-I loop challenge may also be indicative of a protective response; however, this is speculative.

These data demonstrate that oral immunization of rabbits with an SLT-I B-subunit-expressing *V. cholerae* vaccine vector induces a significant neutralizing serum antibody response to SLT-I. The data also suggest the induction of a neutralizing mucosal immune response to the enterotoxic effect of SLT-I in the ileal loop model. Both effects occurred without compromising the production of vibriocidal antibodies or anti-cholera toxin B-subunit antibodies. Our findings suggest the possibility of inducing protective immunity to both the local and systemic complications associated with SLT-producing *E. coli* by oral immunization with an SLT-I B-subunit-expressing cholera vaccine strain. These results encourage the further development and testing of SLT B-subunit-based vaccines.

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RETRACTION

Cloned Shiga Toxin 2 B Subunit Induces Apoptosis in Ramos Burkitt's Lymphoma B Cells

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Volume 70, no. 3, p. 1279–1286, 2002. Subsequent to the publication of this article, we discovered that additional preparations of cloned Shiga toxin 2 (Stx2) B subunit lacked apoptogenic activity in Ramos Burkitt's lymphoma B cells. After exhaustive biological and biochemical characterization, we discovered that the Stx2 B subunit preparations used in our study contained previously undetected Stx2 holotoxin. Since this contaminating Stx2 holotoxin was likely responsible for the apoptogenic activity we attributed to the Stx2 B subunit in this article, we retract the conclusion that the Stx2 B subunit, absent any A subunit activity, initiated apoptosis in Ramos cells. This new finding does not alter our conclusions related to the lack of apoptogenic activity in the Stx1 B subunit preparation or the protective effect of Z-VAD-fmk on protein biosynthesis in the Stx2 B subunit-treated Ramos cells.

Characterization of Shiga-like toxin I B subunit purified from overproducing clones of the SLT-I B cistron

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The cistron encoding the B subunit of *Escherichia coli* Shiga-like toxin I (SLT-I) was cloned under control of the *tac* promoter in the expression vector pKK223-3 and the SLT-I B subunit was expressed constitutively in a wild-type background and inducibly in a *lacI*^{q} background. The B subunit was located in the periplasmic space, and less than 10% was found in the culture medium after 24 h incubation. Polymyxin B extracts contained as much as 160 μg of B subunit/ml of culture. B subunit was purified to homogeneity by ion-exchange chromatography followed by chromatofocusing. Cross-linking analysis of purified native B subunit showed that it exists as a pentamer. In gels containing 0.1% SDS the native protein dissociated into monomers. B subunit was found to have the same glycolipid-receptor-specificity as SLT-I holotoxin. Competitive binding studies showed that B subunit and holotoxin had the same affinity for the globotriosylceramide receptor. We conclude that this recombinant plasmid is a convenient source of large amounts of purified SLT-I B subunit, which could be used for biophysical and structural studies or as a natural toxoid.

INTRODUCTION

The Shiga toxin family is a group of closely related subunit toxins produced by *Shigella dysenteriae* serotype 1 and certain strains of *Escherichia coli* [1–3]. SLT-I-producing and SLT-II-producing strains of *E. coli* have been closely associated with haemorrhagic colitis and the haemolytic uraemic syndrome in humans [4,5], and SLT-IIv has been associated with oedema disease of pigs [1,3,6]. In 1977 Konowalchuk, Speirs and co-workers reported that some strains of *E. coli* produced a cytotoxic effect on vero cells in tissue culture. They partially purified the toxins, which were called verotoxins, and showed that several immunologically distinct variants occurred [7]. It has since become apparent that Shiga-like toxins are identical with verotoxins. For instance verotoxin 1 appears to be identical with SLT-I, and verotoxin E to be identical with SLT-IIv [1,3]. Verotoxin 2 produced by the *E. coli* strain E32511 appears to be very similar to but not identical with SLT-II specified by the bacteriophage 933W [8]. Canadian and British workers continue to use the generic term verotoxin whereas Americans use the generic term Shiga-like toxin (SLT). The toxins consist of an A subunit of 32 kDa associated with B subunits of 7.5 kDa. The toxic activity resides in the A subunit and consists of inactivation of protein synthesis due to an *N*-glycosidase activity that removes the adenine base at position 4324 of 28 S rRNA [9]. The B subunit has been shown by cross-linking studies to be present as a pentamer in the native toxin and is responsible for receptor binding [10]. In the case of Shiga toxins SLT-I and SLT-II the receptor has been identified as globotriosylceramide (Gb_3) [11–14]. In the case of SLT-IIv the primary receptor appears to be globotetraosylceramide (Gb_4), even though this toxin also binds Gb_3 [15]. The coding sequences of both subunits are organized as an operon with the A subunit promoter proximal [16]. In the present paper we report the cloning of the coding sequence for the B subunit of SLT-I under the control of the *tac* promoter. The B subunit was purified and found to exist as a pentamer in the native state, and was found to compete effectively with holotoxin for the Gb_3 receptor.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

E. coli TB1 *lac pro rpsL ara thi* ϕ 80d *lacZ* Δ *M15 hsdR* was obtained from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). *E. coli* JM101 Δ *lac pro supE thi* (F' *traD36 lacZ* Δ *M15 pro AB lacI*^{q}) was obtained from Dr. J. D. Friesen (Department of Medical Genetics, University of Toronto, Toronto, Ontario, Canada). Plasmids pTZ18R [17] and pKK223-3 [18] were obtained from Pharmacia (Uppsala, Sweden).

Plasmid pJLB5 consists of a 3.0 kb *Kpn*I fragment of bacteriophage H19B DNA cloned in the *Kpn*I site of pUC18 [19]; see Fig. 1). Fig. 1 summarizes the construction of the overproducing plasmid. To construct plasmid pJLB34, pJLB5 was cut at the *Bgl*II site and digested with nuclease *Bal*31. The ends were filled with Klenow fragment and dNTPs. The fragment remaining after deletion was cleaved with *Eco*RI, and the piece carrying the SLT-I B cistron was purified by agarose-gel electrophoresis. The fragment was recovered from the gel and cloned into pUC18 cut with *Eco*RI and *Hin*cII. The *Eco*RI–*Hin*dIII fragment was cloned in M13mp18 and its nucleotide sequence was determined. The B cistron coding sequence was recovered from pJLB34 as a 1.1 kb *Pst*I fragment and was then cloned in the *Pst*I site of the polylinker of pKK223-3. Clones with the correct orientation of insertion relative to the *tac* promoter were identified by restriction-endonuclease analysis. One plasmid with the correct orientation was selected and designated pJLB120. pJLB120 was transformed into *E. coli* TB1 for constitutive expression and into

Abbreviations used: SLT-I, *Escherichia coli* Shiga-like toxin I; SLT-II, *Escherichia coli* Shiga-like toxin II; SLT-I B, Shiga-like toxin I binding subunit; Gb_3, globotriosylceramide (Galα1–4Galβ1–4Glc-ceramide); Gb_4, globotetrosylceramide (GalNAcβ1–3Galα1–4Galβ1–4Glc-ceramide; GM_1, Galβ1–3GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glc-ceramide; GM_2, GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glc-ceramide; MAb, monoclonal antibody.

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E. coli JM101 for inducible expression. Bacteria were grown in L-broth [20] or brain heart infusion broth (Difco Laboratories, Detroit, MI, U.S.A.) supplemented as necessary with carbenicillin at 50 μg/ml and isopropyl β-D-thiogalactoside (Bethesda Research Laboratories) at 1 mM.

DNA manipulations

Restriction endonucleases and T_4 DNA ligase were purchased from Boehringer Mannheim Biochemicals (Montreal, Quebec, Canada). Plasmid DNA was prepared by the method of Birnboim & Doly [21]. DNA fragments were purified from low-melt-agarose-gel electrophoresis or polyacrylamide-gel electrophoresis and were recovered with the Geneclean Tm kit (Biocan Scientific, Mississauga, Ontario, Canada) or by electroelution [22] respectively.

Ligations were carried out overnight at 14 °C or at room temperature for 4–6 h, with the use of 2 units of T_4 DNA ligase per 20 μl reaction mixture. Nucleotide sequencing was performed by the Sanger dideoxy chain-termination method [23] with the Sequenase Tm version 2.0 kit from U.S. Biochemical Corp. (Cleveland, OH, U.S.A.).

Expression of SLT-I B subunit

For *E. coli* JM101 (pJLB120), an overnight culture was used to inoculate fresh L-broth supplemented with carbenicillin (50 μg/ml) and was grown to mid-exponential phase (A_{600} = 0.3–0.6) at 37 °C, with shaking at 300 rev./min. Isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM, and incubation was continued with aeration. For *E. coli* TB1 (pJLB120), an overnight culture was used to inoculate fresh L-broth supplemented with carbenicillin (50 μg/ml), and this was grown for 12–18 h at 37 °C, with shaking at 300 rev./min. In both cases the culture was harvested and the pellet was washed once with phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) before extraction.

Polymyxin B extraction

The washed pellet was resuspended in phosphate-buffered saline containing 0.1 mg of polymyxin B/ml in one-quarter of the original culture volume and extracted as previously described [24]. For the purpose of purification, 18 h cultures of *E. coli* TB1 (pJLB120) were extracted with polymyxin B and the extracts were concentrated 10-fold by using a stirred-cell Amicon concentrator with a Ym-5 membrane (Amicon Corp., Danvers, MA, U.S.A.).

Membrane extracts

E. coli TB1 (pJLB120) was grown to mid-exponential phase (A_{600} = 0.4). After the cells had been harvested and the pellet washed with 10 mM-Tris/HCl buffer, pH 8.0, they were resuspended in the same buffer and broken by sonication for 90 s in an MSE Ultrasonic Disintegrator (Johns Scientific, Toronto, Ontario, Canada). Unbroken cells were removed by centrifugation at 7700 *g* for 10 min and the supernatant was centrifuged at 43500 *g* (Sorvall SS34 rotor) for 120 min at 4 °C. The pellet was resuspended in distilled water [25].

Protein determination

Protein determinations were performed with the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL, U.S.A.) according to the method of Smith *et al.* [26], with BSA as standard.

PAGE

SDS/PAGE was performed by the method of Laemmli [27] and Tricine/PAGE according to the method of Schägger & von Jagow [28]. Samples were loaded either in SDS loading buffer [4% (w/v) SDS in 50 mM-Tris/HCl buffer, pH 6.8, containing 2% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol] or in a modified buffer containing no 2-mercaptoethanol. Molecular-mass standards were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.) or Sigma Chemical Co. (St. Louis, MO, U.S.A.). Gels were stained with Coomassie Blue R-250 (Bio-Rad Laboratories) or by the silver stain method of Merril *et al.* [29].

Purification of MAb 13C4

The hybridoma line 13C4 was obtained from the American Type Culture Collection (A.T.C.C. no. CRL 1794) and produces an antibody directed against the B subunit of SLT-I as previously described [30]. Cells were grown in RPMI 1640 medium (Gibco, Burlington, Ontario, Canada) supplemented with 10% (v/v) fetal-calf serum, 10 mM-Hepes and 1 mM-sodium pyruvate in 1-litre spinner flasks (Bellco), and MAb 13C4 was purified from culture supernatants with an Affi-Gel Protein A MAPS II kit (Bio-Rad Laboratories) as recommended by the manufacturer.

Western-blot analysis

Western blots were performed as described by Towbin *et al.* [31]. Protein that had been transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH, U.S.A.) was allowed to react with MAb 13C4. The washed filters were then incubated with peroxidase-conjugated anti-(mouse IgG) antibody (Bio-Rad Laboratories) and developed with chloro-1-naphthol substrate.

Quantification of SLT-I binding subunits

This was performed by a modification of the method of Hawkes *et al.* [32]. Periplasmic extracts of SLT-I B-producing clones, prepared by polymyxin B extraction, were diluted as required and were filtered on to nitrocellulose paper in a slot-blot apparatus (Bio-Rad Laboratories). SLT-I B was detected by using MAb 13C4 according to the Western-blot procedure described above. Blots were scanned with a Molecular Dynamics model 300A computing densitometer. SLT-I B was quantified by comparison with a standard curve generated with purified B subunit protein.

Periplasmic extracts of *E. coli* TB1 containing the vector pKK223-3 with no insert gave a completely negative result in the slot-blot assay. The results of assays of purified B subunit diluted in periplasmic extracts of *E. coli* TB1 (pKK223-3) did not differ significantly from those of the same amount of B subunit diluted in phosphate-buffered saline. There was a significant variation in the assay, which contributed significantly to the large standard deviations in the amount of B subunit detected. Thus when a single sample was assayed ten times the standard deviation was 20% of the mean. Nevertheless when the same sample was assayed on separate days there was good agreement in the results.

Purification of SLT-I B

The concentrated polymyxin B extract was dialysed overnight against 50 mM-Tris/HCl buffer, pH 7.4, and then applied to a DEAE-Sephacel column (1 cm × 20 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.4. Bound material was eluted by using a linear gradient of 0–1 M-NaCl in 50 mM-Tris/HCl buffer, pH 7.4, and 5 ml fractions were collected. Fractions containing SLT-I B were identified, pooled and concentrated with Centriprep-3 concentrators (Amicon Corp.). This pool was dialysed overnight against 25 mM-imidazole/HCl buffer, pH 7.4, and was applied to a column (1.5 cm × 20 cm) of Polybuffer exchanger 94 (Pharmacia) equilibrated with the same buffer. Elution was

carried out with a degassed solution of Polybuffer 74 (Pharmacia) diluted 1:8 with distilled water and adjusted to pH 4.0 with HCl (11 column volumes). Fractions (5 ml) were collected, and the B subunit-positive fractions were pooled and concentrated with Centriprep-3 (Amicon), and ampholytes were removed by means of a Sephadex G-50 gel-filtration column.

Radiolabelling of SLT-I

SLT-I was purified as previously described [24] and iodinated by using Iodobeads (Pierce Chemical Co.) as described by the manufacturer. Two Iodobeads were added to 1 mCi of $Na^{125}I$ in 180 μl of 0.1 M-sodium phosphate buffer, pH 7.2, and were incubated for 5 min. The reaction was initiated by adding 20 μl of SLT-I (1 mg/ml) and was allowed to proceed for 10 min. The labelled protein was separated from unbound I_2 by gel filtration on a Sephadex G-25 column.

Preparation of Gb_3-coated plates

Purified Gb_3, cholesterol and phosphatidylcholine were mixed together in chloroform/methanol (2:1, v/v) in the proportions 2:10:5 (by wt.) and dried down under a stream of N_2. The residue was resuspended in methanol to give a Gb_3 concentration of 1 μg/ml. Portions (100 μl) were dispensed into wells of flexible micro-titre plates (Falcon). The methanol was allowed to evaporate overnight, and the surface of the coated wells was blocked with 2% (w/v) BSA in phosphate-buffered saline for 1 h. The wells were finally washed four times with phosphate-buffered saline containing 0.2% BSA before use.

Competitive binding assay

Serial dilutions of SLT-I (1 mg/ml) or purified B subunit (1 mg/ml) were made in phosphate-buffered saline containing 0.2% BSA and 0.04% NaN_3, and ^{125}I-SLT-I was added to each dilution to give a final value of 10^6 c.p.m./well. After mixing, 100 μl of each dilution was pipetted in triplicate into Gb_3-coated wells and was incubated for 2 h at room temperature. Wells were washed five times with the above buffer, then clipped from the plate and the radioactivities counted on an LKB 1282 γ-radiation counter. The ability of each SLT-I or SLT-I B dilution to inhibit ^{125}I-SLT-I binding was expressed as a percentage of radioactivity bound when no competitor was present (percentage of maximum binding).

T.l.c. binding assays were performed as previously described by Lingwood *et al.* [13].

Gel-filtration (h.p.l.c.) analysis of SLT-I B subunit

A 16 μg portion of purified SLT-I B was injected on to two Waters Protein-Pak 125 h.p.l.c. columns (each 30 cm × 4.6 mm internal diam.) connected in series and eluted at 0.5 ml/min with phosphate-buffered saline. Protein elution was monitored by A_{280} readings. Before the injection of SLT-I B, the columns were calibrated with Blue Dextran (void volume, V_0), BSA, ovalbumin, carbonic anhydrase, cytochrome *c* and aprotinin, all from Sigma Chemical Co. The elution volume (V_e) of each standard was determined and log (molecular mass) plotted against V_e/V_0 to yield a standard curve, from which the apparent molecular mass of SLT-I B could be calculated.

Cross-linking of SLT-I B subunit

B subunit was cross-linked with dimethyl suberimidate as described by Davies & Stark [33]. Cross-linked material was analysed by Tricine/PAGE.

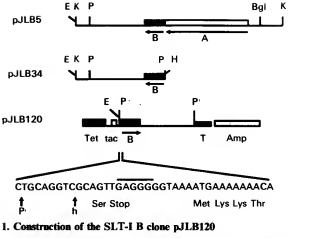

Fig. 1. Construction of the SLT-I B clone pJLB120

Insertion of the B subunit coding sequence into the *Pst*I site of pKK223-3 adjacent to the *tac* promoter is shown. Also indicated are the Shine–Dalgarno sequence (bar above the sequence), the 3′-terminal codon of the A subunit, and the first four codons of the B subunit signal sequence. Horizontal arrows show direction of transcription. Abbreviations: E, *Eco*R1; K, *Kpn*I; P, *Pst*I; Bgl, *Bgl*II; H, *Hin*dIII, h, *Hin*cII; T, rrnb terminator; Amp, β-lactamase gene; Tet, tetracycline-resistance sequence; tac, *tac* promoter.

RESULTS

Construction of the B-subunit-overproducing plasmid pJLB120

The construction is shown in Fig. 1 and is described in the Materials and methods section. *Bal*31 nuclease deletion, fortuitously, gave rise to a clone pJLB34. Sequencing of this clone showed that the deletion ended in the 3′-end of the A subunit cistron. The B subunit coding sequence could be cleaved from pJLB34 as a *Pst*I fragment and was inserted into the *Pst*I site of the expression vector pKK223-3, which contains the *tac* promoter upstream of a polylinker [18]. The sequence of the 5′-end of the *Pst*I fragment was determined in pTZ18R and is shown in Fig. 1. It can be seen that one codon of the 3′-terminus of the A cistron preceded the 12 bp intercistronic gap and that the Shine–Dalgarno sequence and start codon of the B cistron are retained.

Expression of SLT-I B subunit by *E. coli* TB1 and JM101

B subunit was expressed in both strains JM101 (pJLB120) and TB1 (pJLB120), and periplasmic extracts were prepared by using polymixin B. These extracts were concentrated with Centricon-3 ultrafiltration units (Amicon Corp.), and samples were subjected to SDS/15%-PAGE. Fig. 2 shows that both clones expressed the SLT-I B subunit gene, as evidenced by protein bands corresponding to that of purified SLT-I B subunit. In the case of *E. coli* TB1 (pJLB120) the extracts are from cultures grown for 18 h and represent the equivalent of 72 μl of cell culture (lane B). The same quantity of *E. coli* JM101 (pJLB120) extract was loaded in lane C; however, this culture was grown for only 3 h after induction. Confirmation of SLT-I B production was provided by Western-blot analysis of the identical SDS/PAGE gel with the use of MAb 13C4 (lanes F and G respectively).

Efficiency of extraction and localization

The efficiency with which SLT-I B could be extracted by polymyxin B from cells was assessed by means of the slot-blot assay. An overnight culture of *E. coli* TB1 (pJLB120) was harvested, and the pellet was initially extracted with polymyxin B for 1 h, followed by re-extraction for 30 min. After the second extraction the pellet was washed for 30 min with phosphate-

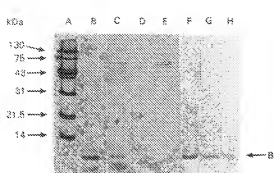

Fig. 2. SDS/PAGE (lanes A–E) and Western-blot analysis (lanes F–H) of periplasmic extracts of *E. coli* TB1 (pJLB120) and *E. coli* JM101 (pJLB120)

Lanes A–E of the gel were stained with 0.1% Coomassie Blue. Lanes F–H of an identical gel were transferred to nitrocellulose paper and allowed to react with MAb 13C4. Lane A, molecular-mass markers; lane B, *E. coli* TB1 (pJLB120); lane C, *E. coli* JM101 (pJLB120); lane D, purified B subunit; lane E, *E. coli* TB1 (pKK223-3), vector control; lane F, *E. coli* TB1 (pJLB120); lane G, *E. coli* JM101 (pJLB120); lane H, purified B subunit. The molecular-mass standards were phosphorylase *b* (130 kDa), BSA (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soya-bean trypsin inhibitor (21.5 kDa) and lysozyme (14 kDa).

buffered saline at 37 °C, and the wash was collected. The supernatant of the harvested culture was also assayed to determine whether SLT-I B was being excreted into the culture medium.

In five separate experiments, the first two polymyxin B extracts yielded SLT-I B at quantities of 80 ± 58 and 48 ± 25 μg/ml of the original culture respectively. More significantly, however, the phosphate-buffered saline wash of the pellet contained residual SLT-I B at 39 ± 32 μg/ml of culture. The mean total yield from polymyxin B extracts and pellet wash was 160 ± 79 μg/ml of culture. The culture .supernatant, when assayed, contained 8 μg/ml of culture, indicating that less than 10% of SLT-I B was being secreted into the culture medium. Since the pellet after extraction still contained relatively high residual quantities of SLT-I B, membrane preparations were examined to determine whether the protein was being trapped in the membrane. The yield from membrane analysis was 0.31 μg/ml of culture.

Production of SLT-I B subunit during the growth cycle

Production of the B subunit by *E. coli* JM101 (pJLB120) and *E. coli* TB1 (pJLB120) was assessed during their growth cycle. At specified intervals portions of the cultures were harvested and the pellets subjected to a single extraction with polymyxin B (0.1 mg/ml). The extracts were then analysed by the slot-blot assay. Fig. 3 shows the quantities of B subunit produced over time for the two strains. The expression of B subunit by *E. coli* TB1 (pJLB120) increased during the first 6 h of growth, corresponding to exponential and early stationary phases. In the case of *E. coli* JM101 (pJLB120), negligible quantities of SLT-I B were produced before induction, after which expression of the protein increased throughout the exponential and early stationary phases. Comparison of the two strains showed that *E. coli* JM101 (pJLB120) expressed greater quantities of SLT-I B than did *E. coli* (pJLB120). For example, after 6 h B subunit in the former was at a level of 100 μg/ml compared with 63 μg/ml in the latter. In addition, both strains demonstrated a decrease in the concentration of B subunit after 24 h. This decrease was sharper in *E. coli* JM101 (pJLB120) than in *E. coli* TB1 (pJLB120).

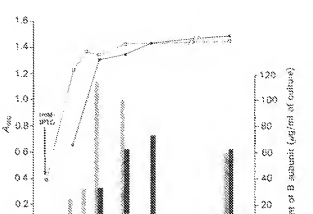

Fig. 3. Quantification of SLT-I B subunit produced in periplasmic extracts of *E. coli* TB1 (pJLB120) and *E. coli* JM101 (pJLB120) during their growth cycle

The amount of B subunit is expressed as μg of B subunit/ml of culture and is represented by stippled bars [▨; *E. coli* JM101 (pJLB120)] and black bars [■, *E. coli* TB1 (pJLB120)]. The A_{650} of the cultures is represented by the continuous line [●—●; *E. coli* TB1 (pJLB120)] and the broken line [□···□; *E. coli* JM101 (pJLB120)]. The arrow indicates the time at which isopropyl β-D-thiogalactoside (IPTG) was added to the *E. coli* JM101 (pJLB120) culture.

Purification

DEAE–Sephacel chromatography of concentrated periplasmic extract yielded a single protein peak containing the B subunit eluted at 200 mM-NaCl (results not shown). Application of the pooled B-subunit-containing fractions to a chromatofocusing column and elution with a pH gradient of 7.4–4.0 yielded a peak that was eluted at pH 5.9 and that contained the B subunit (Fig. 4). Gel filtration of native B subunit showed a single molecular-mass species. Amino acid analysis results (Table 1) showed a single protein species with a composition almost identical with that predicted by the nucleotide sequence.

Purified B subunit appeared to be homogeneous when assessed by silver-stained SDS/PAGE gels (results not shown). When the gel was overloaded and stained with Coomassie Blue it became apparent that most of the material migrated at 7 kDa, while a faint band corresponding to a 15 kDa species was also seen (Fig. 5). The band at 15 kDa was eliminated when the sample was denatured but not reduced. It was suspected that the 15 kDa material represented a dimeric species of B subunit. This was confirmed by Western blotting, which showed that the 15 kDa species reacted with a B-subunit-specific monoclonal antibody PH-1 (Fig. 5).

The molecular mass of native purified B subunit was assessed by h.p.l.c. gel filtration. A calibration curve was generated by plotting log(molecular mass) against V_e/V_t for the standards BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12 kDa) and aprotinin (6.5 kDa). The B subunit was eluted as a single peak at a position corresponding to a molecular mass of 26 kDa, suggesting that it was a multimer. Cross-linking analysis suggested that native B subunit exists as a pentamer (Fig. 6). SDS/PAGE of native B subunit showed that it co-migrated with reduced boiled B subunit, suggesting that the pentamer dissociated into monomers in the presence of 0.1% SDS alone (results not shown).

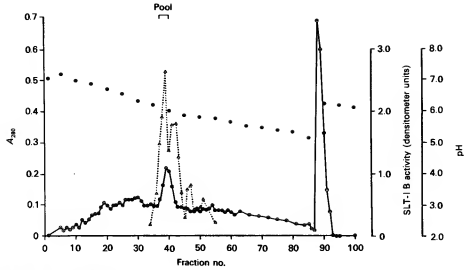

Fig. 4. Chromatofocusing of partially purified SLT-I B

Fractions from DEAE-Sephacel salt elution containing the B subunit were dialysed against equilibration buffer and chromatofocused as described in the Materials and methods section. Fractions (5 ml) were collected, and A_{280} (⊙—⊙), SLT-I B activity (by slot-blot; △···△) and pH (● ●) were determined.

Table 1. Amino acid composition of purified SLT-I B subunit

Amino acid analysis was performed after 24 h hydrolysis of SLT-I B. Abbreviation: N.D., not determined.

	Composition (mol of residue/mol)	
Amino acid	Expected	Observed
Asx	10	10.2
Glx	5	5.8
Ser	3	2.5
Gly	6	6.2
His	1	1.0
Arg	2	2.1
Thr	10	9.4
Ala	2	2.4
Pro	1	1.2
Tyr	2	2.0
Val	6	5.8
Met	1	1.0
Cys	2	N.D.
Ile	3	2.5
Leu	5	5.1
Phe	4	4.0
Lys	5	4.8

Glycolipid binding

In the t.l.c. binding assay, purified SLT-I B had the same binding specificity as SLT-I holotoxin, that is it bound to Gb_3 but not to lactosylceramide, digalactosyldiacylglycerol or Gb_4 (results not shown). In addition, no binding was detected to GM_1, GM_2, monogalactosyldiacylglycerol, galactosylceramide and Forssman antigen (results not shown). To determine whether purified B subunit had the same affinity for Gb_3 as had SLT-1 holotoxin, we used a competitive radiobinding assay. Purified B subunit competed in a concentration-dependent manner with an affinity that was not statistically different from that of SLT-I, as shown by the coincident competition curves in Fig. 7.

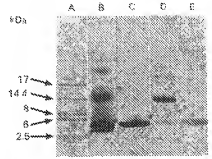

Fig. 5. SDS/Tricine/PAGE (lanes A–C) and Western-blot analysis (lanes D and E) of purified SLT-I B subunit

Samples were dissolved in SDS sample buffer containing 2-mercaptoethanol (lanes A, B and D) or no 2-mercaptoethanol (lanes C and E). Lanes A–C were stained with Coomassie Blue. Lanes D and E were transferred to nitrocellulose paper and allowed to react with the anti-(SLT-I B) monoclonal antibody PH-1 (supplied by Dr. C. Lingwood). The molecular-mass markers (lane A) were myoglobin polypeptide backbone (17 kDa), myoglobin fragments I+II (14.4 kDa), myoglobin fragment I (8 kDa), myoglobin fragment II (6 kDa) and myoglobin fragment III (2.5 kDa).

DISCUSSION

The B subunit of SLT-I has an amino acid sequence identical with that of Shiga toxin [16]. Both have been shown to bind to the glycolipid Gb_3 [11–13], and all the evidence suggests that this is the physiological receptor that mediates the fluid response in rabbit small-intestinal loops [34] and the cytotoxic response in HeLa cells [35] and Daudi lymphoma cells [36]. Furthermore B-subunit-specific monoclonal antibodies have been shown to have neutralizing activity [30]. A synthetic peptide composed of residues of the B subunit of SLT-I/Shiga toxin was shown to induce a neutralizing antibody response in rabbits [37]. Although several investigators have succeeded in producing pure B subunit by dissociating purified holotoxin [10,38,39], the recombinant plasmid described in the present paper provides a convenient source of large amounts. Furthermore the strain is not a

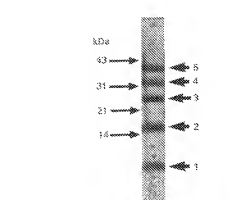

Fig. 6. Cross-linking analysis of SLT-I B subunit

Purified B subunit cross-linked with dimethyl suberimidate was electrophoresed in a 16.5% polyacrylamide gel with Tricine buffer. Arrow 1 indicates the migration of the monomeric species, and arrows 2, 3, 4 and 5 indicate dimers, trimers, tetramers and pentamers respectively. Positions of molecular-mass markers are indicated on the left.

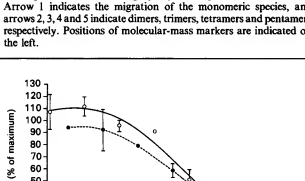

Fig. 7. Competition binding of SLT-I and SLT-I B subunit to Gb_3

Binding of labelled SLT-I in the presence of unlabelled inhibitor is expressed as the percentage of total binding in the absence of inhibitor and is plotted against inhibitor concentration. The curves show inhibition by unlabelled holotoxin (●---●) and by purified B subunit (○—○).

biohazard. B subunit can, then, easily be purified from periplasmic extracts, either as described here, or by the single step P1 glycoprotein affinity chromatography method recently described by Donohue-Rolfe *et al.* [40].

Our results demonstrate that the B subunit is predominantly localized to the periplasmic space, since 10 times more was found in periplasmic extracts than in the culture supernatant. There was a surprisingly large standard deviation when five separate cultures were each subjected to two rounds of polymyxin B extraction followed by one wash in phosphate-buffered saline. Clearly part of the variation was due to the relatively large standard deviation of 15–20% that was found for the slot-blot assay itself. However, it was also clear that the amount of B subunit obtained at each extraction varied considerably. Thus in some cases almost all the B subunit was obtained with the first polymyxin B extraction whereas in other cases the same amount was obtained at each of the two polymyxin B extractions and the phosphate-buffered saline wash. We therefore consider that further efforts to refine and standardize the extraction technique will be important to obtain uniformly high yields. It is also possible that there was significant variation in the production of B subunit in different cultures, but we have not studied this in detail. The results show that induction of expression in the *lacI*^{q} host JM101 with isopropyl β-D-thiogalactoside results in the production of about 50% more B subunit than is produced constitutively in the TB1 host. Since isopropyl β-D-thiogalactoside and a special induction protocol were unnecessary, and the yield was still high, we found it more convenient and less expensive to use the constitutively overproducing strain TB1 (pJLB120). We have consistently been able to obtain 100–120 mg of purified B subunit from 8-litre cultures (J. Gariepy & B. Boyd, unpublished work).

The molecular mass of 26 kDa obtained by gel filtration is clearly lower than expected for a pentamer. Additional biophysical analysis is required to investigate this discrepancy. The cross-linking data show that the SLT-I B subunit exists as a pentamer in the native state in the absence of the A subunit. Our results differ from those reported by Donohue-Rolfe *et al.* [39], who found that B subunit produced by denaturation of holotoxin appeared to exist as a monomer on the basis of cross-linking studies. We believe that their results may have been due to failure to renature the purified B subunit completely. The existence of SLT-I B subunit as a pentamer is similar to the B subunit of cholera toxin [41]. However, native *E. coli* heat-labile enterotoxin and cholera toxin B subunit pentamers are stable in SDS/PAGE [41], whereas even non-reduced native SLT-I B subunit migrated as a monomer in the presence of SDS in our studies. We wondered whether association with the A subunit would modulate the binding specificity or affinity of the B subunit pentamer. Our results clearly show that the specificity for the Gb_3 receptor is retained by the B subunit. Similarly, the competition studies show that the affinity for Gb_3 is virtually identical. This suggests that purified B subunit could be used as a convenient non-toxic marker to examine the routing of toxin in animal models and as a reagent to identify toxin-binding sites in tissue sections *in vitro*. It will also be interesting to see whether purified B subunit can be used as a natural toxoid for immunization. Migasena *et al.* have demonstrated that the B subunit of cholera toxin in combination with killed whole vibrios, when given orally, was immunogenic [42]. It is conceivable that such immunization could be used to prevent haemolytic uraemic syndrome, especially in Argentina, where the incidence appears to be very high [43].

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